Docket No.: 20722 US (C038435/0175476)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
Beat FLÜHMANN *et al.*	)	Examiner: K.E. Weddington
Serial No.: 10/766,118	)	Art Unit: 1614
Filed: January 27, 2004	)	
For: **PHYTANIC ACID DERIVATIVE COMPOSITIONS AND METHOD OF TREATING AND/OR PREVENTING DIABETES MELLITUS**	)	

DECLARATION OF DR. BEAT FLÜHMANN UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Beat Flühmann, a citizen and resident of Zürich, Switzerland, hereby declare as follows:

1. I am a Swiss Federal Qualified Pharmacist from the Federal Institute of Technology (ETH) Zurich, Switzerland; in addition I have a PhD from the Department for Biology of the Federal Institute of Technology (ETH) Zurich; Switzerland.

2. I am currently employed by DSM Nutritional Products Europe Ltd. From 1998 to April 2005 I was working in the Research Center for Human Nutrition and Health in the Vitamins Division of F. Hoffmann-La Roche Ltd which became Roche Vitamins Ltd before it was taken over by DSM and became DSM Nutritional Products Ltd. The main

focus of my work during this period was on dietary ingredients for the prevention of metabolic diseases such as diabetes and obesity.

3. I am a co-inventor of the invention described and claimed in the captioned application entitled "PHYTANIC ACID DERIVATIVE COMPOSITIONS AND METHOD OF TREATING AND/OR PREVENTING DIABETES MELLITUS."

4. The present application discloses and claims methods for treating or preventing non-insulin dependent diabetes mellitus (NIDDM) by administering to a human or an animal an effective dose of a pharmaceutical composition or a dietary supplement comprising from about 0.1 to about 1000 mg of phytanic acid or a derivative of phytanic acid.

5. I am aware that an Office Action has issued with regard to the present application on March 1, 2005. (Paper No. 20050224.) It is my understanding that in the Office Action, the Examiner asserted that the methods of claims 10-13 were unpatentable for containing subject matter which was not described in the specification in a such a way as to enable one skilled in the art to make and use the invention. (*Id.* at 3.) In particular, the Examiner asserted that "[t]here are no examples showing the phytanic acid, phytanic acid precursors or derivative of phytanic acid will, in fact, prevent non-insulin dependent diabetes mellitus especially in a human or an animal not presently at risk of or predisposed to developing such a disease or disorder." (*Id.* at 6.)

6. I must respectfully disagree with the Examiner's conclusion. As I demonstrate below, the specification combined with the knowledge in the art would have sufficiently enabled one of skill in the art to practice the present invention. Moreover, the

experimental results submitted herewith further confirm what is disclosed in the specification at page 5, line 29 - page 6, line 5 and page 10, lines 1-23 and in Examples 2-4 that phytanic acid, at physiological concentrations, acts via both peroxisome proliferator-activated receptors (PPAR) α and γ to activate the transcription of a distinct pattern of genes that favors glucose uptake. Moreover, phytanic acid can be used to manage insulin resistance. These *in vitro* and *in vivo* results confirm what is disclosed in the specification, namely that phytanic acid produces a biochemical result, which prevents or mediates the metabolic abnormalities associated with NIDDM *in vivo*.

7. As stated in the specification:

> [The] experiments show that phytanic acid derivatives, preferably phytanic acid, can increase and stimulate the transcription of the genes for glucose transporters and glucokinase resulting in increased glucose uptake in hepatocytes.
>
> Moreover, phytanic acid derivatives normalize and increase the glucose level without a concomitant risk of hypoglycemia and is thus excellently suited for the treatment or prevention of diabetes mellitus. Page 5, line 29 - page 6, line 5.

8. Moreover, the specification states:

> The present invention demonstrates that glucose uptake in primary cultures of hepatocytes is markedly induced by levels of phytanic acid derivatives one order of magnitude higher than serum concentration. The enzymes involved in glucose uptake are the various members of the facilitative glucose transporter (GLUT) family, GLUT-1, GLUT-2, and GLUT-4 and glucokinase. The up regulation of the mRNA levels of GLUT-1 and GLUT-2 observed in the present invention are accompanied by increased 2-deoxy-D-glucose uptake. The liver type glucose transporter GLUT-2 is distinguished from the other GLUT isoforms by being a low-affinity glucose transporter with high turnover rate.
>
> The presence of a low affinity glucose transporter ensures that in liver, glucose flux is directly proportional to the

plasma glucose concentration. Moreover, in hepatocytes GLUT-2 is coupled with the regulated phosphorylating activity provided by the glucokinase. Thus, during states of glycogen synthesis, glucokinase is up regulated and can increase the formation of intracellular glucose-6-phosphate, maintaining a low intracellular concentration of free glucose. Page 10, lines 1-23. (Citations omitted.)

9. The Examples show:

- Measurement of 2-deoxy-D-glucose uptake in primary rat hepatocytes cultures during the time course of study, revealed a substantial increase of 2-deoxy-D-glucose uptake in hepatocytes treated with 100 μM phytanic acid as compared with the control, palmitic acid or DHA. Example 2, page 16, lines 1-6 and Figure 2.

- Measurement of mRNA levels for GLUT-1 and PEPCK in response to 100 μM phytanic acid revealed after 6 hours a maximal 5.6 (5.1-6.2)-fold and 4.4 (3.4-5.7)-fold induction, respectively. mRNA levels of GLUT-2 and glucokinase were found to be maximal after 24-h stimulation period with phytanic acid. A 2.2 (1.6-2.9)-fold and 2.4 (1.7-3.3)-fold induction of mRNA levels for GLUT-1 was observed in hepatocytes stimulated for 24 h with 100 μM phytanic acid and palmitic acid, respectively. ... Treatment of the cells for 24 h with phytanic acid (0.01-100 μM) induced mRNA levels for GLUT-2 at least 2-fold, with a maximal induction of 3.2 (2.7-3.8)-fold (100 μM). ... mRNA levels of glucokinase and PEPCK were induced by 100 μM phytanic acid 3.0 (2.5-3.4)-fold and 2.5 (1.7-3.6)-fold. Example 3, page 19, line 25 - page 20, line 14 and Figures 3-5.

- The application of different concentrations of phytanic acid could reduce the plasma insulin. Example 4, page 20, lines 31-32 and Figure 7.

10. To confirm what was already disclosed in the specification, experiments were conducted under my supervision to demonstrate that phytanic acid mediates the up-regulation of glucose transporter gene expression and glucose influx in rat hepatocytes and to demonstrate that the administration of phytanic acid to animals (mice) leads to

significantly lower plasma glucose levels, and therefore, a delayed onset of NIDDM in animals fed a high-fat diet.

***IN VITRO* ANALYSIS**

Expression and Reporter Plasmids

11. The plasmids pSG-PPAR-alpha, -beta, and -gamma expressing human PPAR-alpha, -beta and -gamma$_1$, respectively, were provided by Roger Clerc and Markus Meyer of F. Hoffmann-La Roche Ltd., Basel, Switzerland.

12. The plasmids were constructed by cloning full length coding sequences of the pertinent receptors into the pSG5 expression vector. The (ACO-PPRE)$_4$-tk-luc reporter plasmid was provided by Hugo Stirnimann of Roche Vitamins Ltd., Basel Switzerland. Four repeats of the PPRE from the acyl-CoA oxidase (ACO) gene promoter (CCGGACCAGGACAAAGGTCA) followed by a tk minimal promoter were cloned into the pGL-3 basic vector.

Transient transfection and reporter gene assays

13. Cells were transiently transfected using LipofectAMINE™ 2000 Reagent in Opti-MEM® I (both from Life Technologies Inc., Basel, Switzerland). After a 24 hour incubation, the cells were trypsinised. 75 μl aliquots of the cell suspension in DMEM supplemented with 0.1% (w/v) fatty acid-free BSA and 2 mM L-Glutamine (Stimulation Medium) (~25,000 cells) were then added per well to an opaque 96-well tissue culture plate and incubated for 4-5 hours to allow the cells time to adhere to the bottom of the

wells. To stimulate the cells, 25μl of 4x-concentrated ligands in stimulation medium were added to each well in triplicate, and the plate was incubated for 24 hours.

14. To assay the luciferase activity on each plate, 75 μl of Steady-Glo® Luciferase Buffer (Promega Corp., WI, USA) were added to each well. The plate was incubated for 15 minutes at room temperature on a plate shaker. Luminescence was then measured with a Luminoskan luminescence reader (Labsystems, Helsinki, Finland) for 5 seconds per well.

Transcriptional activation of PPAR isoforms by phytanic acid in CV-1 cells

15. We investigated the transactivational effect of phytanic acid and its natural stereomers on the $(ACO\text{-}PPRE)_4$-tk-luc construct, co-transfected with the human PPAR isoforms alpha, beta and gamma in CV-1 cells. Endogenous ACO gene expression, as well as expression of comparable reporter constructs, has previously been shown to be ligand dependently activated by PPARs. Dreyer, C. *et al., Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors.* Cell, 1992. 68(5): p. 879-87. No effect on cell viability was observed in all cell lines used with up to 300 µM phytanic acid for 24 hours .

16. CV-1 cells were stimulated for 24 hours in the presence of 30 μM phytanic acid.

17. Other CV-1 cells were stimulated for 24 hours in the presence of palmitic acid and DHA as well as the ligands Wy-14643, PGA-1 or ciglitazone, specific for the receptor isoforms PPAR-alpha, -beta and -gamma, respectively, at 30 μM (except for

Wy-14643, PGA_1, and Ciglitazone, which were at 50 μM, 50 μM, and 10 μM respectively).

Results

18. A 24 hour stimulation of CV-1 cells with 30 μM phytanic acid revealed a significant stimulation of luciferase activity in cells either co-transfected with pSG-PPAR-alpha, - beta or - gamma; whereas the phytanic acid precursors *trans*- or *cis*-phytol, had no or very low transactivational potential. Phytanic acid showed a stronger selectivity towards PPAR-alpha and - gamma as compared to PPAR beta.

19. Figure 1, below, shows ACO-PPRE-driven luciferase transactivation in the stimulated CV-1 cells. The results of the (3RS,7R,11R) phytanic acid treatment were compared to those obtained from treatments with palmitic acid and DHA as well as the ligands Wy-14643, PGA-1 or ciglitazone, specific for the receptor isoforms PPAR-alpha, -beta, and -gamma respectively. The results shown are from a representative experiment. Each experiment was carried out three times independently, and each measurement made in triplicate. Fold inductions were calculated from RLUs relative to the unstimulated situation. Error bars are shown as +S.D.

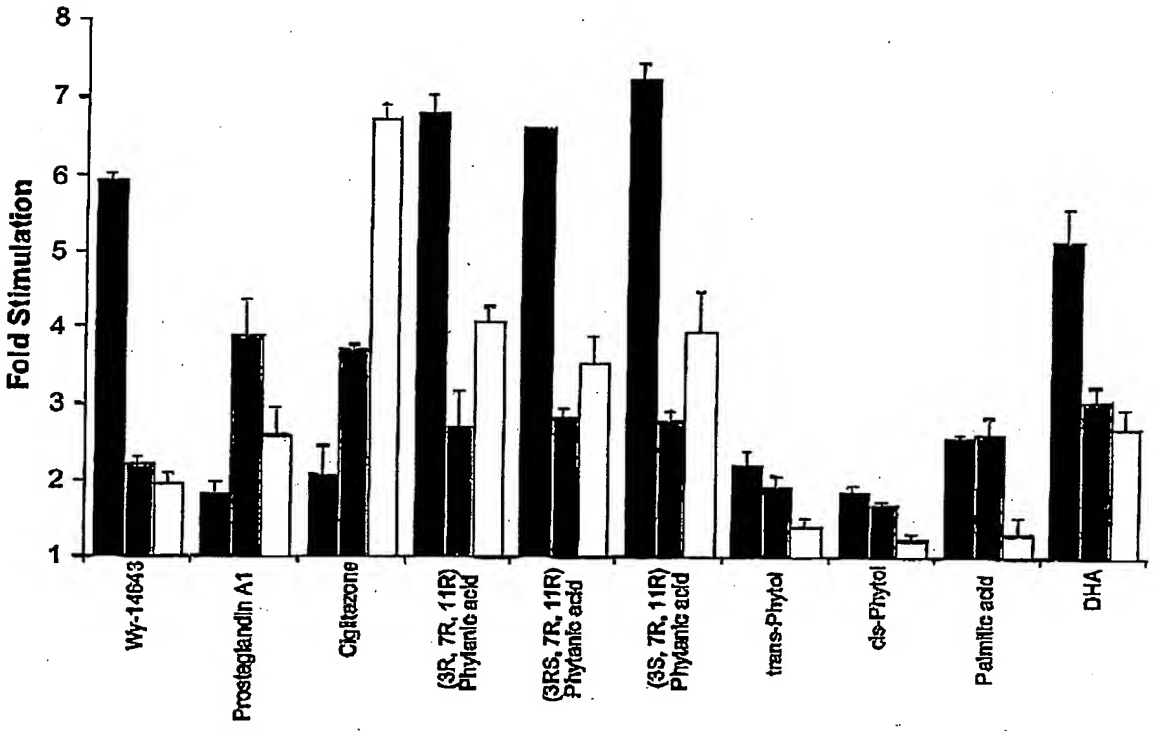

Figure 1. ACO-PPRE-driven luciferase transactivation in stimulated CV-1 cells.

20. In addition, a dose-dependent stimulation of the PPAR isoforms was conducted as described above with varying concentrations. CV-1 cells were stimulated for 24 hours with (3R,7R,11R) phytanic acid, (3RS,7R,11R) phytanic acid, (3S,7R,11R) phytanic acid, and the following standard compounds Wy-14643, and ciglitazone for PPAR-alpha and - gamma, respectively.

21. Figures 2a and b, below, show the concentration dependent activation of PPARs (Figure 2a - PPAR alpha and Figure 2b - PPAR gamma): (3R,7R,11R) phytanic acid – diamonds, (3RS,7R,11R) phytanic acid – squares, (3S,7R,11R) phytanic acid – triangles, and Wy-14643 and ciglitazone for PPAR-alpha and - gamma, respectively – circles. Curves were calculated using KaleidaGraph 3.5 (SynergySoftware).

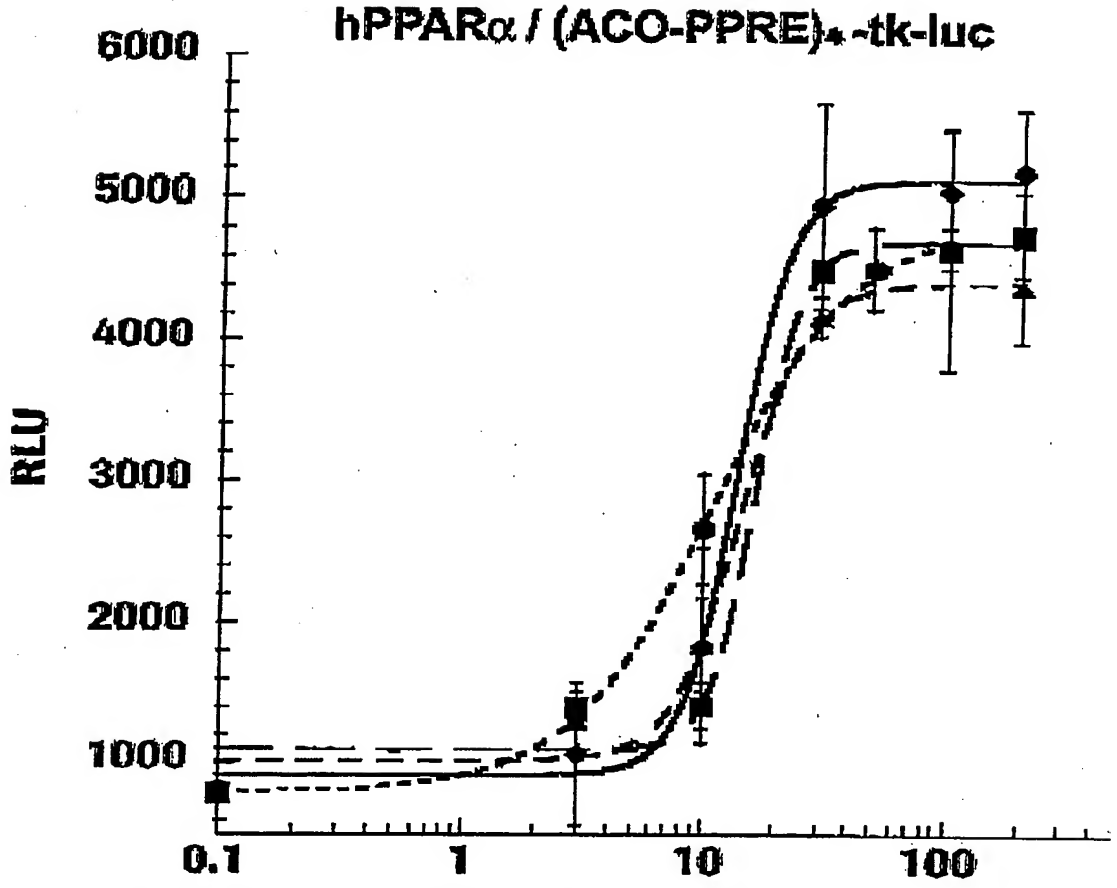

Figure 2a. Concentration dependent activation of PPAR alpha.

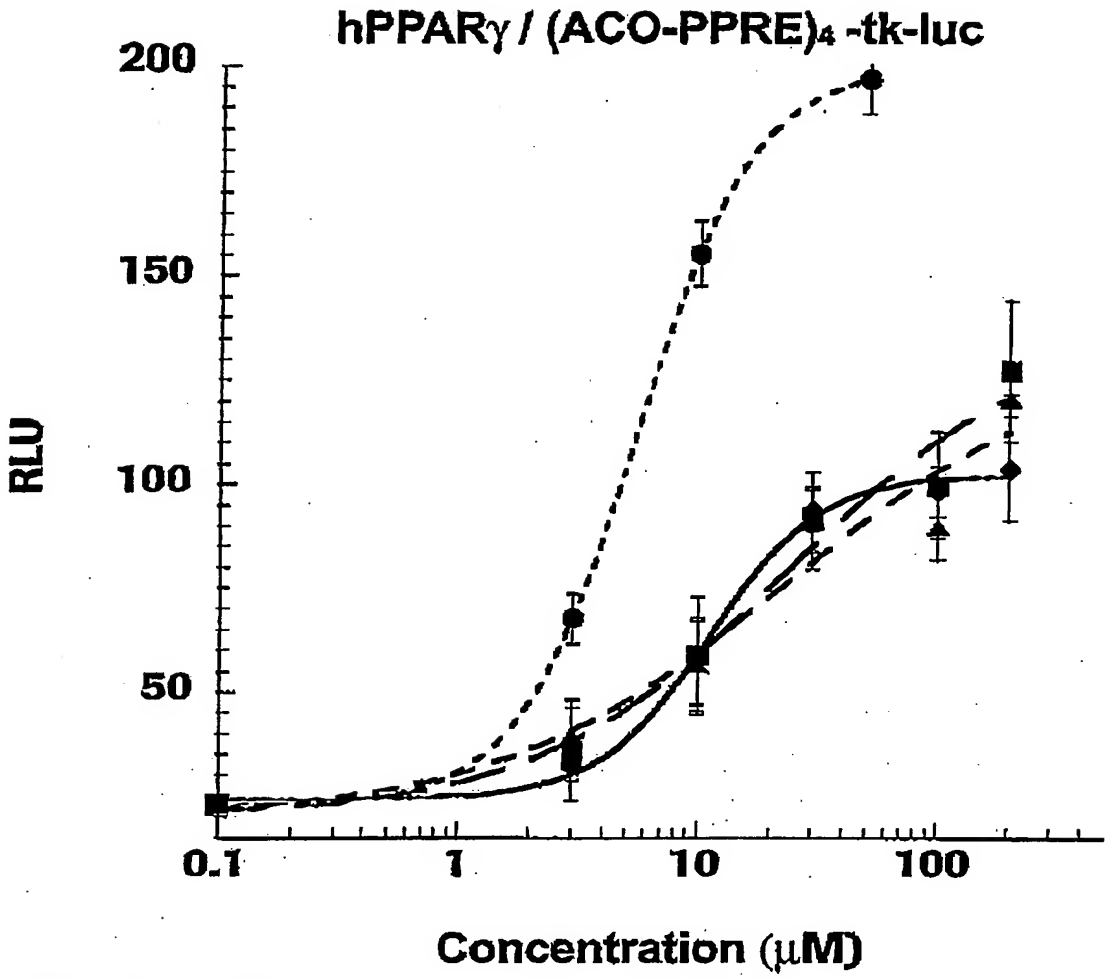

Figure 2b. Concentration dependent activation of PPAR gamma.

22. I conclude from Figures 2a and b that the dose-dependent stimulation of the PPAR isoforms with (3R,7R,11R)- or (3S,7R,11R) phytanic acid revealed no significant difference in transactivation as compared to the mixture thereof (Figure 2).

IN VIVO ANALYSIS

Animals

23. Sixty male C57BL6 mice were obtained from Iffa Credo (Lyon, France) at about 7 weeks of age. The mice were housed four per cage in a humidity- and temperature-controlled (23 –24°C) facility with a 12-12-hour light-dark cycle for the duration of the study.

24. After 2 weeks of acclimatization on a mouse chow diet, the mice were matched by body weight (bw) and assigned to one of the four treatment groups with 12 mice per group (9 wks of age). One group of mice was maintained on the chow diet (lean control (LC) group). The other groups were switched to a Western type of high-fat diet, containing on a weight basis 20.4% of calories as fat and 45% as sucrose alone (fat control (FC) group), or with phytanic acid at two different concentrations, 75 mg/kg bw (PYA75) or 150 mg/kg bw (PYA150)

High fat / high sucrose feed mouse model for NIDDM prevention

25. At weeks 0, 6, 10, 19, and 23 of intervention, a drop of blood was obtained from the tail vein after 15 hours of food deprivation. Blood glucose was measured using a glucose meter (MediSense® Precision Q I D®, Abbott Laboratories, Illinois, USA).

26. To demonstrate the NIDDM preventive potential of phytanic acid, the progression of the fasted blood glucose concentrations was measured in a diet induced NIDDM mouse model (DIO).

Statistical Analysis

27. Data are presented as mean ± SEM. Two-way ANOVA was used to analyze the effects of dietary intervention and baseline blood glucose concentrations (covariate). The outcome variable was the change from baseline (value at wk 0) for each mouse, with diet as the main factor.

28. Dunnett's test was used to test for differences between groups with FC mice as the control group. The covariate "baseline blood glucose concentrations" was coded as the difference between baseline blood glucose for each animal and the mean baseline for all animals.

29. The interaction between main factor and covariate was also analyzed. If the interaction term was not significant, it was removed and the statistical analysis was re-run with the reduced model. Significance for the main factor diet was only examined if the multiple R^2 for the model was significant ($p< 0.05$). All statistical analyses were conducted with a α level of 0.05, using SPSS (version 905 for Windows, Chicago, IL, USA). One-tailed p values were used to evaluate diet effects, while two-tailed p values were used for the covariate and interaction term.

Results

30. Figure 3 shows changes from day 0 in fasting plasma glucose levels in the C57BL/6J mice. Values are means ± SEM (n=12). * $p<0.05$ as compared with FC.

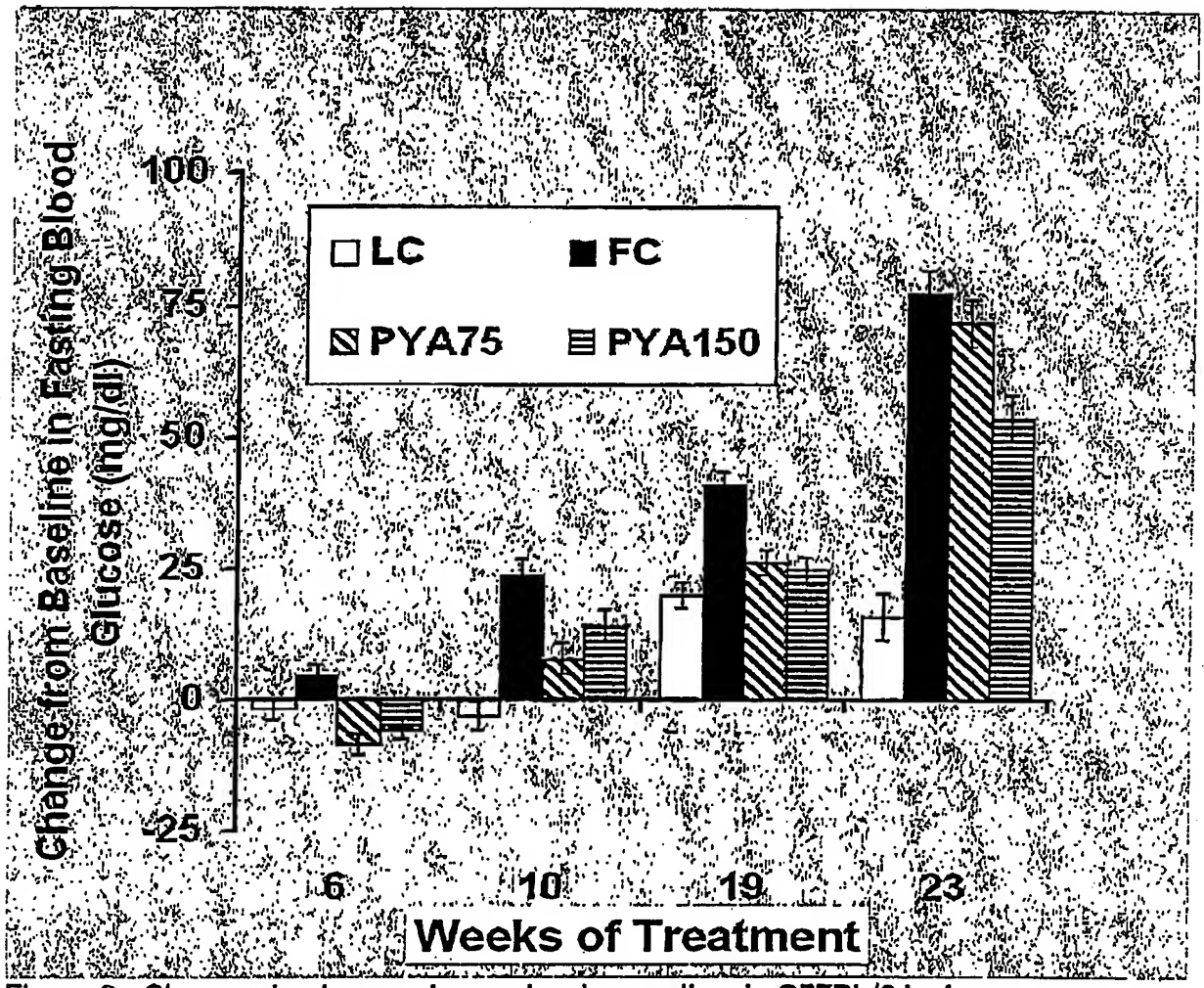

Figure 3. Changes in plasma glucose levels over time in C57BL/6J mice.

31. After 6 weeks of phytanic acid administration, the change from baseline in fasted blood glucose concentration was significantly higher in FC mice than in the LC ($p=0.013$) and in PYA75 mice ($p=0.016$). Although the change from baseline was also higher in FC mice than in PYA150, the difference did not reach statistical significance.

32. At 10 weeks, fasted blood glucose increased further in FC mice, and it was higher than in any other group. However, the FC mice were only statistically different from the LC ($p<0.0001$), PYA75 ($p=0.034$).

33. At week 19, the change in FC mice was significantly higher than in any other group ($p<0.0001$ for LC, $p=0.012$ for PYA150, and $p=0.017$ for PYA75).

34. By week 23, mice receiving 75 mg/kg bw phytanic acid (PYA75) were no longer different from FC. However, the FC mice remained statistically different from the LC ($p<0.0001$), and PYA150 ($p=0.023$).

CONCLUSION

35. Specific agonists for the Retinoic X Receptor (RXR) and PPAR alpha as well as gamma have been reported to be effective in lowering hyperglycemia in rodents. Mukherjee, R. *et al.*, *Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists.* Nature, 1997. 386(6623): p. 407-10. In human intervention studies, using PPAR gamma ligands, insulin sensitizing and plasma glucose lowering effects were observed. In contrast, there are no comparable studies reported showing these effects with RXR ligands in man. In human intervention studies gemfibrozil, a PPAR alpha agonist, does not affect glycaemic control. Vuorinen-Markkola, H. *et al.*, *Lowering of triglycerides by gemfibrozil affects neither the glucoregulatory nor antilipolytic effect of insulin in type 2 (non-insulin-dependent) diabetic patients: Effects of gemfibrozil on low-density lipoprotein particle size, density distribution, and composition in patients with type II diabetes.* Diabetologia, 1993. 36(2): p. 161-9. Accordingly, the RXR and/or PPAR alpha agonistic potential can not be considered as an indicator of a compound's anti-diabetic property. Although the precise molecular basis for the insulin sensitizing effect is not fully understood, it is widely accepted that the agonists activity on PPAR gamma is essential for efficacious treatment and

prevention of NIDDM in man. Lehmann, J.M. *et al., An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma).* J Biol Chem, 1995. 270(22): p. 12953-6.

36. Phytanic acid has been previously described as a ligand for both RXR and PPARα. *See, e.g.,* Kitareewan, S. *et al., Phytol metabolites are circulating dietary factors that activate the nuclear receptor RXR.* Molecular Biology of the Cell, 1996. 7(8): p. 1153-66; Lemotte, P.K., S. Keidel, and C.M. Apfel, *Phytanic acid is a retinoid X receptor ligand.* Eur. J. Biochem., 1996. 236(1): p. 328-33; Wolfrum, C. *et al., Phytanic acid is ligand and transcriptional activator of murine liver fatty acid binding protein.* J. Lipid Res., 1999. 40(4): p. 708-14; Ellinghaus, P. *et al., Phytanic acid activates the peroxisome proliferator-activated receptor alpha (PPARalpha) in sterol carrier protein 2- / sterol carrier protein x-deficient mice.* J. Biol. Chem., 1999. 274(5): p. 2766-72; and Zomer, A.W. *et al., Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferator-activated receptor alpha.* J. Lipid Res., 2000. 41(11): p. 1801-7. As demonstrated above, phytanic acid is an agonist for PPAR-alpha as well as PPAR-gamma. This is in contrast to the findings with other fatty acids tested, which have only an agonistic effect on PPAR-alpha. Based on these novel and surprising findings, I conclude that phytanic acid is useful for prevention and treatment of NIDDM in man.

37. As shown above, mice fed on a high fat diet develop NIDDM characterized by elevated glucose levels. In animals fed phytanic acid, the increase of plasma glucose levels was significantly lower compared to control animals. Therefore, a delayed onset

of NIDDM was observed in animals fed with phytanic acid. From this finding, I conclude that phytanic acid is useful for the prevention of NIDDM.

38. Based on my knowledge and experience, a review of the relevant knowledge in the art, and the disclosure set forth in the present specification, it is my opinion that one skilled in the art would readily recognize that phytanic acid would be useful for treating or preventing non-insulin dependent diabetes mellitus. Moreover, the experimental results described above further confirm what was disclosed in the specification at page 5, line 29 - page 6, line 5 and page 10, lines 1-23 and in Examples 2-4, namely that administration to a human or an animal of an effective dose of a pharmaceutical composition or a dietary supplement containing phytanic acid, a phytanic acid precursor, or a derivative of phytanic acid would be effective to treat or prevent non-insulin dependent diabetes mellitus.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: July 20, 2005

Beat Flühmann